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SCIENTIFIC PAPER

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OPTIMAL GROWTH OF *Saccharomyces cerevisiae* (PTCC 24860) ON PRETREATED MOLASSES FOR THE ETHANOL PRODUCTION: THE APPLICATION OF THE RESPONSE SURFACE METHODOLOGY

Saccharomyces cerevisiae (PTCC 24860) growth on pretreated sugar beet molasses was an optimized via statistical approach. In order to liberate all monomeric sugars, pretreated sugar beet molasses with dilute acid was obtained. The influence of process parameters such as sugar concentration, nitrogen source, pH and incubation time on the cell growth were investigated by a design expert software with the application of a central composite design (CCD) under response surface methodology (RSM). The optimal culture conditions were pH of 5.3, incubation time of 24 h and medium composition of 35 g reduced sugars, 1.5 g NH_4Cl and 1 g yeast extract per liter of the media. At optimal cell growth conditions and incubation time of 12 h, the maximum ethanol production of 14.87 g/L was obtained.

Key words: optimization; ethanol; pretreated molasses; Saccharomyces cerevisiae; fermentation; response surface methodology.

Microorganisms as biocatalysts are widely used in fermentation processes where a product and biomass are obtained from the fermentable sugars [1,2]. These biologically active organisms have significant contributions in beverage and food industries [3]. *Saccharomyces cerevisiae* is a budding yeast known as a baker yeast or brewer yeast. This microorganism is most often used in the fermentation process and obtains energy from various carbon sources. Yeasts are the most common microorganisms for ethanol fermentation. Among the yeast kingdom, *S. cerevisiae* is one of the well known ethanol producers [4,5]. Ethanol is an essential chemical which is used as a raw material for a vast range of applications including chemicals, fuel (bioethanol), beverages, pharmaceuticals and cosmetics [2,6]. The bioconversion of glucose resulted in ethanol production *via* Embden-Myerhoof--Paranas pathway [2,7]. *S. cerevisiae* has short germination time and is easily cultured in large scale processes [8]. The rapidly growing S. cer*evisiae* ensured the ethanol production in batch culture with the limited substrate concentration (50 g/L); the ethanol production with high substrate concentrations and without substrate inhibition was reported by the immobilized cells [4].

For economical reasons, researchers have paid special attention to various sources of raw material for fermentation industries [8]. Molasses is an agro-industrial waste and a by-product of the sugar industry which has a noticeable amount of monomeric and polymeric sugars [9,10]. In fact, molasses is a dark brown, thick solution which is obtained from the final stages of crystallization. It is widely used in chemical industries to produce baker yeast and ethanol. Upgraded and pretreated molasses are the biggest economical source of carbohydrate for ethanol fermentation [9]. Molasses contained reduced polymeric sugars that can further be treated to form monomeric fermentable sugar by diluted acid; glucose is a predominant sugar produced from the pretreated sucrose in raw carbon resources [11].

The growth of an organism is strongly influenced by medium composition and the ethanol production is cell dependent. Thus, the optimization of the growth media composition and cultural parameters is the main task in a fermentation process [12]. To meet the optimal cell growing demands, it is necessary to improve the performance of the system and thus increase the

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ethanol yield without increasing the cost of the production [13]. Limitations of one-at-a-time-parameter optimization can be eliminated by employing Response surface method (RSM) which is used to explain the combined factors in a fermentation process [14,15]. Generally, RSM defines the effect of the independent process variables, alone or in combinations, and generates a mathematical model that describes the entire process [16,17]. Also, the RSM summarizes mathematical methods and statistical inference for an approximate functional relationship between a response variable and a set of design variables [18]. The most popular RSM is the Central composite design (CCD) which is an efficient and flexible technique that provides sufficient information on the effects of process variables and overall experimental error with a minimum number of experiments [19].

The purpose of the present work was to maximize the cell growth of *S. cerevisiae* in batch culture using a pretreated molasses solution (PMS) as a carbon source. A statistical analysis was applied for the optimum cell growth. In RSM, the effects of four independent variables such as PMS and NH_4Cl concentration, pH and incubation time were investigated. At optimum growth conditions, experiments were conducted for the ethanol production.

EXPERIMENTAL

Pretreatment of molasses

Sugar beet molasses was obtained from Shirvan (Khorasan, Iran) sugar factory. The characteristics of the molasses are summarized in Table 1. The concentrated molasses was diluted with distilled water to adjust the reduced sugar concentration to 100 g/L (220 ml of concentrated molasses diluted with 780 ml of distilled water). The sugar concentration was determined by DNS method [20]. Diluted HCl acid solution was added to molasses in a ratio of 1/400 for the purpose of pretreatment and to increase the reducing sugar content of the molasses solution [5]. After 1 day, the pretreated dilute molasses solution was autoclaved at 121 °C for 15 min and the final measured sugar concentration was 255 g/L. The pH of the pretreated molasses solution (PMS) was at acidic condition (pH 3.78). The pH of medium was neutralized with 2 M sodium hydroxide solution.

Table 1. The characteristics of molasses obtained from Shirvan Sugar Factory

Color	Dark brown
Density, g/L	1380
Ashes, mg/L	0.12
TSS (Total suspended solids), mg/L	0.83
Initial sugar concentration, g/L	515
Final pretreated sugar concentration, g/L	1025

Seed culture preparation

The microorganism *S. cerevisiae* (PTCC 24860) for fermentation was obtained from Persian Culture Collection, Tehran, Science Organization of Science and Technology. The yeast was grown in a medium consisting of glucose, yeast extract, NH_4Cl, peptone, KH_2PO_4, NaCl, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$: 15, 3, 2, 1, 0.1, 0.08, 0.07 and 0.01 g/L, respectively. The initial pH of the medium was adjusted to 5. In addition, the inoculated media was incubated at 30 °C in an incubator-shaker (Stuart, UK) with the agitation rate of 190 rpm for duration of 24 h.

Culture preparation and growth conditions

The media contained sugar beets molasses as the carbohydrates concentration in the range of 15-35 g/L, with increments of 5 g/L and NH_4Cl as a nitrogen source in the range of 1-5 g/L with increments of 1 g/L. The initial pH of the medium in the range of 4.4 to 5.6 with increments of 0.3 was studied. The basal medium contained supplementary compounds such as yeast extract, KH_2PO_4, NaCl, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$ and buffer $C_8H_5KO_4$: 1, 0.1, 0.08, 0.07, 0.01 and 10.2 g/L, respectively. Potassium hydrogen phthalate ($C_8H_5KO_4$) was used as a buffer solution to adjust the pH of the media. A 50 mL of the prepared media was transferred to a series of 250 mL Erlenmeyer flasks. The sterilized and inoculated media was incubated and agitated in an incubator-shaker maintained at 190 rpm and 30 °C [4]. The samples were analyzed for cell growth, substrate and product concentrations.

Growth measurements (cell optical density)

In batch fermentation, about 30-35% of carbon source is converted to the cell population while ethanol yield is 50% of carbohydrates [21]. The remaining sugar source is used for ATP generation and cell maintenance. Cell optical density was monitored to determine the cell growth and the samples were drawn in every 2 h time interval. The sample size of 1 ml was drawn from the inoculated medium for analysis of the cell growth and substrate utilization. In order to determine the cell growth or cell optical density (OD), the light absorbance of each sample was measured by spectrophotometer (Unico, USA) at wavelength of 620 nm. The known volume of the samples with defined OD was filtered (0.45 μm, Sartorius, Biotech, Germany) and the cell dry weight (CDW) was measured based on the developed calibration curve. The CDW was proportional to the cell optical density. For

initial investigation of the cell growth and sugar utilization, a 100 ml medium was prepared in a 250 ml Erlenmeyer flask which consisted of molasses with total sugar; yeast extract, NH_4Cl, peptone, KH_2PO_4, NaCl, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$: 30, 3, 2, 1, 0.1, 0.08, 0.07, 0.01 g/L, respectively. The prepared media was autoclaved and then inoculated with 5% of seed culture. The culture was incubated in an incubator-shaker maintained at 190 rpm and 30 °C.

Ethanol analysis

A gas chromatograph (Philips PU4400, UK) equipped with a flame ionization detector (FID) and data acquisition system with computer software (Claritylite 4.2, Data Apex, Czech Republic) was used to analyze the ethanol concentration. The installed column was PEG 20 M (glass column) 1.5 m and 1/8 mm (Philips, USA). Temperature programming was implemented for the liquid sample analysis. During the analysis, the column temperature was initially maintained at 120 °C; 2 min later, the oven temperature was increased at a rate of 10 °C/min until it reached 150 °C. The injector and detector temperatures were 150 and 200 °C, respectively. The flow rate for carrier gas (Nitrogen) was set at 30 ml/min. A solution of 2-methyl-1-butanol (1%, v/v) was used as an internal standard with the concentration of 50 μL/mL of the sample. The injection sample volume was 1 μL. In each set of experiments, the data points were repeated in triplicates and the mean value was reported.

Response surface method (RSM)

The response surface method (RSM) was used to define the optimum condition for the cell growth. The RSM consists of empirical correlations for evaluating the relations between a cluster of controlled experimental factors and measured responses according to one or more selected criteria. The effects of four parameters including sugar from the pretreated molasses, NH_4Cl, pH and incubation time were investigated.

Design Expert 7.0 software (Stat-Ease, Inc., Minneapolis, MN, USA) was used for experimental data analysis. A design of 30 experiments was formulated for four factorial (2^4) and six replicates at the central points. The second-order polynomial model was employed. The value of the dependent response was the mean value of three independent experiments. The optimum values of the selected variables were obtained by analyzing the response surface plots. Each of the parameters was coded at five levels from the lowest, medium low, medium, medium high and highest values: −2, −1, 0, +1 and +2, respectively [22]. In addition, statistical analysis of the model was performed by the analysis of variance (Anova).

RESULTS AND DISCUSSION

Ethanol fermentation is known as a growth associated process, in other words, in the presence of active cells the rate of the ethanol production has reached the maximum value while the cell concentration has reached a stationary phase [2,21].

Cell growth

The logistic equation for the cell growth is described by the following equation [2,21,23,24]:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = \mu_{\mathrm{m}}\left(1-\frac{x}{x_{\mathrm{m}}}\right)x \tag{1}$$

where μ_{m} is the maximum specific growth rate (h^{-1}) and x_{m} is the maximum attainable biomass concentration (g/L). The integration of the biomass production rate with the use of the initial condition (at $t = 0$, $x = x_0$) gives a sigmoidal variation of x as a function of t which may represent both an exponential and a stationary phase, Eq. (2):

$$x = \frac{x_0 e^{\mu_{\mathrm{m}} t}}{1-(x_0 / x_{\mathrm{m}})(1-e^{\mu_{\mathrm{m}} t})} \tag{2}$$

The experimental data was described by a logistic model. Matlab 7.1 was used to incorporate experimental data for curve fitting. The maximum specific growth rate, μ_{m} and x_{m}, maximum bio mass concentration were 0.74 h^{-1} and 2.534 g/L, respectively. The coefficient for the fitted data with the Logistic model was R^2 = 0.991. The cell growth curve and sugar concentration profiles with respect to incubation time are shown in Fig. 1. The solid line represented the logistic model well fitted with the experimental data. The last datum point may be affected by the cell death phase. Four distinct phases were defined on a growth curve such as lag (2 h), exponential (2-14 h), stationary (14-24 h) and death phases (> 24 h). At the stationary phase, the maximum cell concentration and sugar conversion were 2.83 g/L and 90%, respectively. The death phase was developed after 24 h of incubation. Since the growth curve clearly showed that the death phase starts after 24 h of fermentation, then any sample drawn from batch culture within 24 h should contain viable cells. Therefore, the assumption for the CDW data which represent the viable cells in batch fermentation is valid.

Response surface analysis

The process variables were investigated in order to achieve a realistic model. It was perfectly under-

stood from experimental data that the ethanol production depends on the cell growth rate in fermentation. The effects of the sugar concentration, NH_4Cl, pH and incubation time (each variable in five levels) on the growth of *S. cerevisiae* were investigated. The concentrations of PMS and NH_4Cl, pH and incubation time varied according to the experiment strategy (lowest, medium low, medium, medium high and highest values). The values for the sugar concentration (15--35 g/L), NH_4Cl as nitrogen source (1-5 g/L), pH (4.4--5.6) and incubation time (6-30 h) were the corresponding five coded levels for each of the independent variables. The values of CDW (cell dry weight) were obtained by conducting 30 experiments and also the data were predicted by RSM.

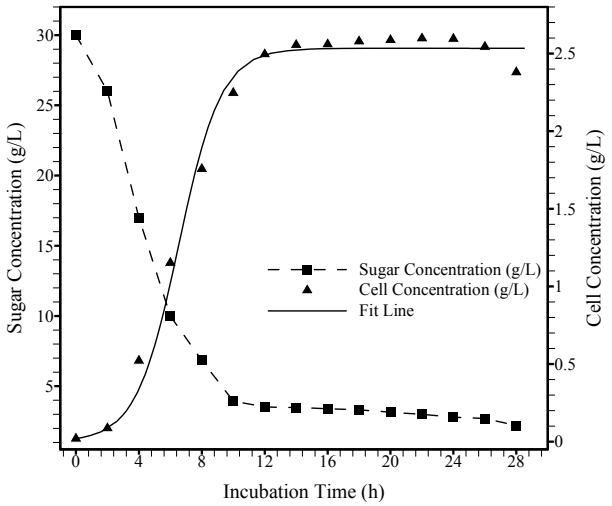

Figure 1. Sugar concentration profile and Saccharomyces cerevisiae growth curve: (▲) cell concentration; (■) sugar concentration.

The *F* and *P*-values show the significance of each coefficient. The *F*-value is the measurement of variation of the data about the mean. The high *F*-value and very low probability indicate that the model is in good prediction of the experimental results. The model *F*-value (37.16) and *P*-value (<0.0001) indicated that the model terms were significant (Table 2). The accuracy of the fit of the model was checked by multiple correlation coefficients (R^2). In this case, the statistical significance of the method was confirmed by the multiple correlation coefficient of 0.972. A reasonable agreement between the predicted value of the multiple correlation coefficient (pred. R^2 = 0.842) and the value of the adjusted multiple correlation coefficient (adj. R^2 = 0.946) indicated a good consistency between the experimental and predicted values for CDW [13]. A relatively low value of the coefficient of variance (*CV* = 6.35%) showed a significant pre and reliability of the experiments.

The three dimensional response surface plots are presented in Fig. 2 to illustrate the main and interactive effects of the independent variables on CDW.

Figure 2a shows the effect of the sugar concentration in PMS and NH_4Cl on the cell growth at pH 5 and the incubation time of 24 h. Both variables had a high linear effect on the cell growth. According to the obtained data, the quadratic effect of PMS was not high (*P* = 0.2104), but NH_4Cl had a partially significant quadratic influence on CDW (*P* = 0.0729). The *P*-value (0.1914) for the interaction of these two variables (PMS and NH_4Cl) was low. The concentration of carbohydrate plays a major role; with the increase of the carbohydrate concentration, the cell growth rate in the fermentation broth also increased. Since the sugar concentration in the PMS in the range of 15-35 g/L was low, there was no substrate inhibition in the fermentation. The maximum cell growth was obtained with the PMS concentration of 35 g/L. In a medium with the stated amount of PMS, the optimum NH_4Cl concentration was 2.12 g/L. At defined optimum conditions, the maximum CDW of 3.05 g/L was obtained.

Figure 2b shows the effect of PMS and pH on the cell growth in a medium containing a fixed amount of nitrogen source (NH_4Cl, 1 g/L) with an incubation time of 24 h. The presented data for the analysis of the cell growth and ethanol production rates showed some physiological significance on ethanol fermentation. According to the experimental data, the pH range of 4.4-5.6 was selected for the cell growth and ethanol production. The pH had high linear effect on CDW (*P* = 0.0022), but the quadratic effect of this factor was not significant (*P* = 0.1505). The low *P*-value (0.001) showed a significant interaction between pH and the carbon source; each of these two parameters had the influence on CDW the most when the other factor was at the lowest level. At the carbohydrate

Table 2. Analysis of variance (Anova) for the response surface quadratic model (adequate precision as signal to noise ratio: 25.766)

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Probability (*P*) > *F*
Model	8.34	14	0.60	37.16	<0.0001
Residual	0.24	15	0.016	-	-
Pure error	0.0059	5	0.00118	-	-
Total	8.58	29	-	-	-

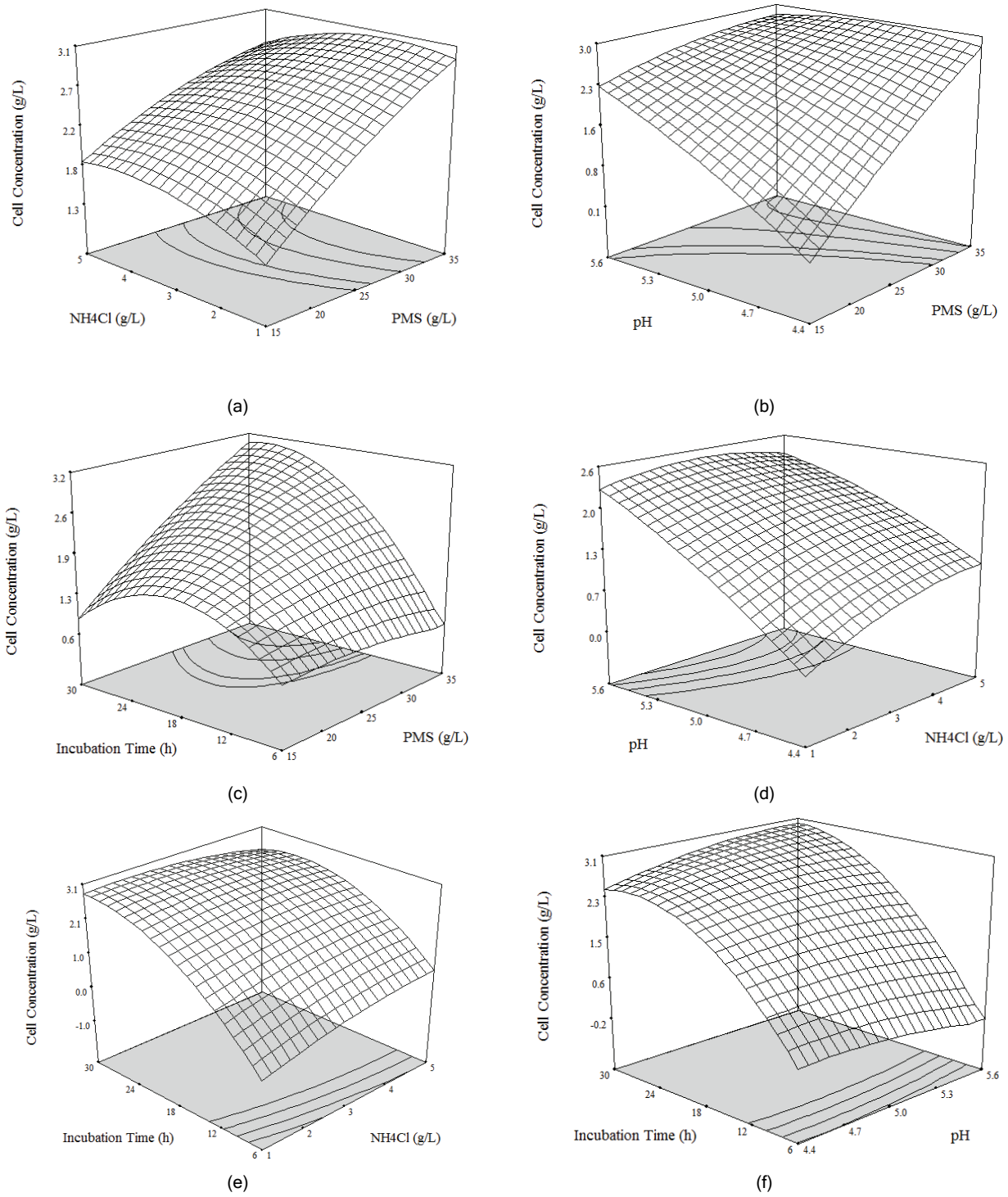

Figure 2. Response surface plots for the interaction of a) PMS with NH_4Cl on CDW, b) PMS with pH on CDW, c) PMS with incubation time on CDW, d) NH_4Cl with PH on CDW, e) NH_4Cl with incubation time on CDW and f) PH with incubation time on CDW.

concentration of 35 g/L and pH of 5, the maximum CDW (2.94 g/L) was predicted by a regression analysis.

The effect of incubation time and PMS with a fixed concentration of the nitrogen source (NH_4Cl, 4 g/L) and pH value of 4.7 has been investigated. As shown in Fig. 2c, an increase in incubation time up to an optimum value resulted in the increase of CDW, but the extended period of incubation might lead to the reduction of pH and toxic byproducts which cause cell death. At low incubation time, low substrate concentration was more suitable for the high cell growth

and the increase of incubation time resulted in the increase of the optimum substrate concentration. When the incubation time varied from 6 to 30 h, the optimum PMS concentration shifted from 20 to 35 g/L. The high interaction between these two parameters is also confirmed by *P*-value (0.0003). The two low *P*-values of linear and quadratic terms for the incubation time showed high linear and quadratic effects of this parameter on the response. The maximum CDW (3.08 g/L) was predicted at the carbohydrate concentration of 35 g/L at the incubation time of 26 h.

Figure 2d describes the variation of pH and nitrogen source for maximum cell growth while the PMS concentration (15 g/L) and incubation time (24 h) were fixed. The *P*-value (0.1364) for the interaction of these variables indicated low interaction. Therefore, pH had insignificant influence on the optimum NH_4Cl concentration. The optimum NH_4Cl concentration, pH and maximum CDW were 2.8 g/L, 5.6 and 2.45 g/L, respectively.

The effect of incubation time and NH_4Cl on CDW with fixed PMS concentration (30 g/L) and pH value of 5 is illustrated in Fig. 2e. At optimum conditions (NH_4Cl, 2.6 g/L and the incubation time, 24 h), the maximum CDW of 2.88 g/L was obtained.

The effect of pH and incubation time on CDW with fixed concentrations of PMS (30 g/L) and NH_4Cl (2 g/L) are shown in Fig. 2f. The low *P*-value (0.0639) shows a significant interaction. It has been reported that, *S. cerevisiae* is able to produce organic acids [25]. A long period of incubation may lead to higher concentrations of acids and thus reduces the media pH which has a negative impact on the cell growth. Therefore, for the process with a long incubation time, it is recommended to start with a high pH media. As the incubation time was increased from 6 to 30 h, the optimum pH slightly shifted from 4.4 to 5.6. Maximum cell concentration of 3 g/L was obtained at optimum conditions: incubation time (30 h) and pH (5.6).

The estimated values of PMS, NH_4Cl, pH and incubation time for maximum CDW were 35, 1.5 g/L; 5.3 and 24 h, respectively. At these optimum levels, an experiment was carried out and the CDW of 2.92 g/L was obtained, which was very close to the value (2.98 g/L) predicted by the central composite design (CCD).

Ethanol production

At optimum growth conditions for *S. cerevisiae* in PMS, influential process parameters on the ethanol production were investigated. The experiments were carried out in order to study the effects of various operation parameters such as: incubation time, concentration of NH_4Cl, pH and sugar concentration on the ethanol production. In each set of experiments, one parameter was varied and the rest were kept constant to determine the optimum value of the investigated parameter. The achieved optimum parameter in each step was used in the next experiment. The obtained results are shown in Fig. 3.

Figure 3a shows the effect of incubation time on the ethanol production and CDW. When the cells are in an exponential phase, these cells have maximum activity. Based on the cell growth curve, 12 h incubation time showed that the ethanol concentration was progressively increased to maximum value of 14.87 g/L. The other three sets of experiments were conducted with 12 h incubation time. At the beginning of the stationary phase, the highest ethanol concentration was performed where the cell propagation and death rates were identical. Then, the ethanol concentration gradually decreased. That was due to sugar depletion, ethanol oxidation and organic acid formation for the long incubation time (24 h) [25]. The accumulation of ethanol in fermentation broth caused deactivation of alcohol producing enzymes [26].

Figure 3b shows the effect of the NH_4Cl concentration on CDW and the ethanol production. With initial NH_4Cl concentration of 1.5 g/L, maximum CDW of 2.93 g/L was obtained. Also, at the initial NH_4Cl concentration of 1.5 g/L, the ethanol concentration of 14.1 g/L was achieved. It was reported that molasses may contain a considerable amount of minerals and nitrogen sources and the additional nitrogen sources may have negative impact on the ethanol production [6]. Therefore, the excess amount of nitrogen sources associated with ammonium ions may retard the growth rate that might cause an inhibitory effect, which consequently resulted in decreases of CDW and the ethanol production.

The effect of media pH variation on the ethanol production was also investigated. When the ethanol concentration in the fermentation broth has reached the highest value, then ethanol was utilized and organic acids were formed [25]. The intermediate byproducts in the ethanol fermentation pathway (most probably organic acids) may cause pH reduction in the fermentation media [21]. At pH value of 5.6, the maximum ethanol production was achieved (Fig. 3c).

Figure 3d presents ethanol and CDW production with respect to the initial sugar concentration in the media. The sugar concentration was in the range of 35 to 60 g/L. With the initial sugar concentration of 55 g/L and the incubation time of 12 h, the highest value of the ethanol production was 18.3 g/L.

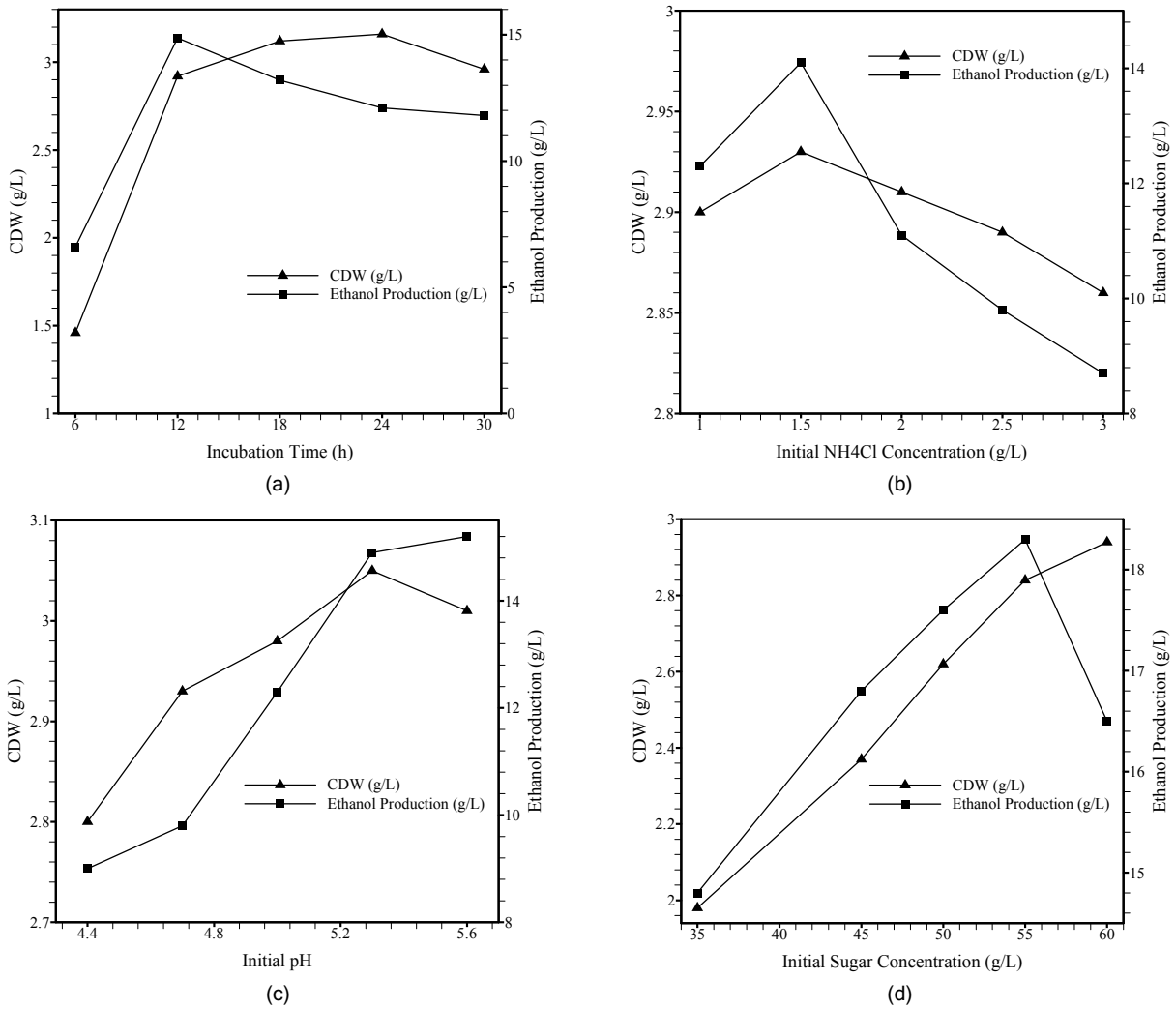

Figure 3. Effect of incubation time, NH_4Cl, pH, initial sugar concentration on ethanol production; (▲) CDW, (■) ethanol production.

CONCLUSION

The conclusion was that the central composite design estimated the optimum conditions for *S. cerevisiae* growth well. The optimum values were 35 g/L PMS, 1.5 g/L NH_4Cl, pH 5.3 and the incubation time of 24 h for maximum CDW (2.97 g/L). With the additional nitrogen source, the maximum amount of ammonium chloride (5 g/L), a negative impact on the trend of the cell growth was observed. It was also concluded that the prolonged incubation time had reduced the pH of the media. When the incubation time was prolonged, the concentration of ethanol was depleted. Most probably, *S. cerevisiae* oxidized ethanol in the fermentation broth and also the other end products which caused the media pH shift to a slightly acidic condition. The growth model developed in the fermentation media was based on the obtained data; results proved that the ethanol production rate was growth-associated. Finally, the maximum ethanol production (14.87 g/L) was obtained at 12 h incubation time.

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NAUČNI RAD

OPTIMIZACIJA RASTA *Saccharomyces cerevisiae* (PTCC 24860) NA PRETRETIRANOJ MELASI ZA PROIZVODNJU ETANOLA PRIMENOM METODE ODZIVNE POVRŠINE

Rast Saccharomyces cerevisiae (PTCC 24860) na pretretiranoj melasi iz šećerne repe je optimizovan primenom statističkog pristupa. U cilju oslobađanja svih monomernih šećera, melasa je tretirana razblaženom kiselinom. Uticaj parametara procesa, kao što su: koncentracija šećera, izvor azota, pH i vreme inkubacije na rast ćelija je statistički procenjen pomoću softvera Design Expert i aplikacijom centralnog kompozitnog dizajna (CCD) i metode odzivne površine. Optimalni uslovi gajenja su bili: pH 5,3, vreme inkubacije 24 h i sastav podloge od 35 g redukujućih šećera, 1,5 g NH_4Cl i 1 g ekstrakta kvasca po litri podloge. Pri optimalnim uslovima rasta ćelija i vremenu inkubacije 12 h ostvarena je maksimalna proizvodnja etanola od 14,87 g/L.

Ključne reči: optimizacija; etanol; melasa; Saccharomyces cerevisiae; fermentacija; metoda odzivne površine.